DEVELOPMENT OF PEPTIDE ANTIBIOTIC-BASED CONTROL STRATEGIES FOR *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted from July 1, 2003 to October 31, 2003.

ABSTRACT
In this project, we are evaluating the effectiveness of peptide antibiotics such as cecropins A, B, and P1 against *Xylella fastidiosa*. In our initial experiments, we have found that cecropins (in particular cecropin A) are effective at inhibiting the growth of *X. fastidiosa*. We are currently attempting to express individual cecropins in an in vitro expression system based on baculovirus expression vectors and insect cells. The baculovirus system will allow us to easily manipulate the coding sequence of the peptide antibiotic in order to optimize their efficacy. In the second phase of our project we will attempt to express the most effective cecropin constructs in transgenic plants and determine whether (i) active cecropin is produced and (ii) if active cecropin is expressed, does feeding by GWSS on these transgenic plants reduce *X. fastidiosa* transmission.

INTRODUCTION
Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop the growth of bacteria. Antibiotic inhibition of *Xylella.* (at least 17 isolates tested) has been analyzed for six different antibiotics (ampicillin, kanamycin, neomycin, penicillin, streptomycin, and tetracycline) (4, 10). These studies demonstrate that antibiotic treatment is potentially an effective method for the control of *X. fastidiosa*. Under field conditions, however, barriers between the antibiotic and bacterium, and degradation effects will require significantly higher application doses than those found effective in the laboratory. Such doses may be impractical especially for broad-spectrum antibiotics due to secondary effects (e.g., toxicity against mammalian red blood cells) and the risk of increasing resistance. Thus, although traditional antibiotics such as tetracycline are highly active, an effective delivery system to bring them in contact with *X. fastidiosa* in the plant or insect vector is not available.

Recently, a great deal of scientific effort is being put into the study of a second type of antimicrobial agent called peptide antibiotics. Peptide antibiotics have been identified from a wide range of organisms including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals. In general, peptide antibiotics are small (less than 50 amino acids), have a net positive charge, and are composed of 50% or more of hydrophobic amino acids (6, 18). One class of peptide antibiotic is composed of so-called ribosomally synthesized peptides (5). These peptides are encoded by single genes and synthesized by a protein complex (ribosome) that is found in all cells and processed following synthesis via common pathways (6, 13). In other words, unlike traditional antibiotics, peptide antibiotics have the potential to be easily produced by common protein expression systems or in transgenic organisms (e.g., plants). Furthermore, because peptide antibiotics are “gene-based”, they can be produced directly at the location where they are needed and their synthesis can potentially be regulated by using appropriate gene promoters.

Some of the best-characterized peptide antibiotics are the cecropins. Cecropins were the first peptide antibiotics to be identified in an animal, the giant silkmoth *Hyalophora cecropia* (9, 17). At least ten different cecropins have been isolated from lepidopteran (moths and butterflies) and dipteran (flies) insects (1, 12) and one cecropin has been identified from the small intestine of a pig (11). Cecropins are composed of a single chain of 35-39 common L-amino acids and do not contain disulfide bonds (12). Cecropins are active against many Gram(-) bacteria and some Gram(+) bacteria, but are inactive against eukaryotic cells at concentrations that are antimicrobial (1, 7, 18) and possibly at concentrations up to 300 times higher (17). *X. fastidiosa* is a Gram(-) bacterium (8). In Gram(-) bacteria the antibacterial activities of cecropins A, B, and P1 are up to ten-times greater than tetracycline (1, 2). Cecropins have a unique combination of characteristics (specificity, gene basis, small size, potency against Gram(-) bacteria, etc.) that should make them ideal substances for the control of *X. fastidiosa* in GWSS.

A basic component of Integrated Pest Management (IPM) is the use of cultural practices such as the purposeful manipulation of the environment in order to reduce pest damage (14). This concept is elegantly illustrated in the southern San Joaquin Valley where strips of alfalfa are planted between cotton fields for protection against a key cotton pest, the lygus bug. This practice is successful because the lygus bug prefers feeding and living on alfalfa over cotton (15). The pest species is

effectively trapped in the "trap crop" because of a particular preference and damage to the crop is minimized. In the case of a grape vineyard and Pierce's disease (PD), other plantings adjacent to the grape vineyard may serve as preferred food source and breeding ground for the glassy-winged sharpshooter (GWSS) (16). The goal of our proposed project will be to develop a model system to test the potential of a transgenic, peptide antibiotic-expressing trap plant for the control of *X. fastidiosa* transmission by GWSS. Our approach is highly unique in that we are attempting to reduce transmission frequency through the use of a transgenic trap plant rather generating a transgenic crop plant (i.e., transgenic grape). Perhaps a direct manifestation of this objective would be to generate transgenic, cecropin-expressing grape. We believe, however, that this "direct" approach has several drawbacks including longer generation times, unknown effect on the quality of the fruit or wine, regulatory concerns, and public acceptance. Thus, our approach should be faster in terms of field applicability. However, once the genetic technology is optimized for the trap plant it can later be applied to the target grape crop if there is such a need. Furthermore, should our approach be successful for PD, it will also be applicable to other insect-vectored, bacterial diseases.

OBJECTIVES

1. Identify peptide antibiotics (cecropins) that are effective against *Xylella fastidiosa.*
    i. Determine the antibiotic sensitivity of *X. fastidiosa* to chemically synthesized cecropins.
    ii. Produce modified cecropins using baculovirus expression vectors.
    iii. Determine the toxicity of cecropins against GWSS cells grown in culture.
2. Analyze the effectiveness of cecropins produced in transgenic *Arabidopsis.*
    i. Generate transgenic Arabidopsis expressing cecropin that is active against *X. fastidiosa.*
    ii. Determine the localization, yield, activity, and stability of plant-expressed cecropin.
    iii. Analyze the effect of cecropin expression on the transgenic *Arabidopsis.*
    iv. Analyze the effectiveness of plant-expressed cecropin for the control of *X. fastidiosa* transmission.

RESULTS AND CONCLUSIONS

Objective 1

During the four month-long reporting period, we have established procedures for the continual culture and storage of *X. fastidiosa* in our laboratory. In general, our procedures are copied from protocols established in the Bruce Kirkpatrick laboratory. We have tested GYE (glutamate yeast extract) (3), PD3, and PW media for their abilities to support maximal growth of *X. fastidiosa*. In our hands, we have found that PD3 gives the fasted growth of *X. fastidiosa* (Temecula strain) in liquid medium (roughly 20- and 135-fold increases in the OD_{600} at 7 and 14 days post inoculation, respectively) and on agar plates (formation of a light lawn by 10 days post seeding).

Using the optimized growth conditions, we have examined the minimal inhibitory concentrations (MIC assay) at which cecropins A, B, and P1 are effective in inhibiting the growth of *X. fastidiosa* (see Table 1). On the basis of our preliminary experiments, cecropins A, B, and P1 appear to be effective at partially inhibiting *X. fastidiosa* growth at concentrations of 0.05, 0.25, and 0.5 μM, respectively, at two weeks post inoculation. In general, cecropin A appeared to be the most effective against *X. fastidiosa*. The effectiveness of the cecropins as well as kanamycin was reduced by three weeks post inoculation. This was speculated to be the result of degradation. We are currently repeating the preliminary experiments with intermediate concentrations of cecropin and chemical antibiotics.

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Table 1. Effect of Cecropin or Kanamycin on *X. fastidiosa* Growth

	Concentration (μM)	% Increase in bacterial concentration in comparison to cultures lacking antibiotic		
		Week 1 (% ± s.d.)	Week 2 (% ± s.d.)	Week 3 (% ± s.d.)
cecropin A	0.5	69 ± 3	47 ± 47	64 ± 42
	0.25	72 ± 10	80 ± 21	117 ± 5
	0.1	103 ± 13	68 ± 2	87 ± 25
	0.05	110 ± 46	50 ± 1	91 ± 22
cecropin B	0.5	69 ± nd	114 ± 6	87 ± 45
	0.25	63 ± 31	75 ± nd	110 ± 15
	0.1	72 ± 101	128 ± 63	90 ± nd
	0.05	93 ± 17	101 ± 18	74 ± 10
cecropin P1	0.5	98 ± 18	70 ± 40	70 ± 62
	0.25	82 ± 18	98 ± nd	120 ± 17
	0.1	111 ± 52	93 ± 24	72 ± 24
	0.05	93 ± 10	99 ± 22	73 ± 18
Kanamycin	2	11 ± 3	9 ± 8	16 ± 2
	1	19 ± 8	32 ±39	33 ± 22
	0.5	42 ±16	77 ± 9	103 ± 16
	0.25	60 ± 13	72 ± 17	105 ± 12

nd = not determined

FUNDING AGENCIES
Funding for this project was provided by the University of California Pierce's Disease Grant Program.

EFFECTS OF SUB-LETHAL DOSES OF IMIDACLOPRID ON VECTOR TRANSMISSION OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted from August 2003 to October 2003.

ABSTRACT
Two agents, soil-applied imidacloprid (Admire 2F) and particle film (Surround™ WP) foliar sprays were applied to grape seedlings to test their effect on vector insects' (glassy-winged sharpshooter, GWSS, and blue-green sharpshooter, BGSS) host preference, under caged laboratory conditions. Imidacloprid at sub-lethal dosages that inhibit feeding performance did not show any repellency effect against either of the insects, but GWSS on the imidacloprid-treated plants moved more frequently. In contrast, the particle film strongly repelled BGSS (GWSS not tested yet), but the insects seldom changed location once they landed and settled on a plant with or without the treatment. The test plants used in the preference tests will be diagnosed for the Pierce's disease bacterium (*Xylella fastidiosa*), in order to evaluate how the above results affect transmission.

INTRODUCTION
Chemical treatments often are used to control plant diseases caused by insect vector-borne pathogens (Perring et al. 1999). Insecticides have proven effective to reduce numbers of glassy-winged sharpshooter (GWSS). The systemic insecticide imidacloprid (Admire 2F, Bayer Co., Kansas City, MO) has been studied in relation to GWSS mainly for its mortality effect (Bethke et al 2001). Although the insecticide seems to be effective in killing GWSS, a recent study in Georgia showed that the imidacloprid application slowed the rate of PD spread but was prone to the high infestation of GWSS (Krewer et al 2001). Therefore, it is important to know how imidacloprid's effect on the insect's behavior affects the pathogen transmission process. The probability of vector transmission depends not only in the number (*n*) of vectors per plant, but on what percentage are infective (*i*), how efficiently infective insects transmit (*E*), and the time spent on a plant (*t*) (Purcell 1981). Imidacloprid has been shown to affect the feeding behavior (Bethke et al 2001) of GWSS and behavioral effects on other vector insects (Quintela and McCoy 1997), so we examined how GWSS transmission of *X. fastidiosa* might be affected by systemic imidacloprid in grape. This knowledge could be useful in optimizing the use of imidacloprid or suggest alternative/supplemental strategies.

The dosage of imidacloprid applied in this experiment was "sub-lethal", which had less than 10% mortality over 24 hour period, as determined from our previous experiments to sharply reduce GWSS' feeding. We tested whether this deterred feeding would repel the insects from imidacloprid-treated grapevines. For comparison, a similar experiment using the particle film (Surround™ WP, Engelhard Corp., Iselin, NJ) was also conducted, as field and laboratory studies have already demonstrated the repellency of particle films for GWSS (Puterka et al 2003).

OBJECTIVES
1. Observe and compare how the Admire 2F or Surround WP application affects a grapevine's acceptance as a host by GWSS and BGSS.
2. Evaluate the above effects on the transmission rate of PD.

RESULTS AND CONCLUSIONS
Preference Test Settings
We set 8 arenas (W60xL60xH45 cm) covered with white mesh, each with 2 plants, inside in a greenhouse insectary. There were 3 different combinations of grape seedlings (*Vitis vinifera*, Pinot Noir): 2 arenas each of No-choice (+/+, both treated) and No-choice (-/-, both untreated), and 4 arenas of Choice (+/-, one treated and one untreated). The treatments applied here were either imidacloprid (Admire 2F, 0.000197gAI/500g soil applied 7 days prior to the experiments) or Surround WP (6%, sprayed for full coverage the day before the experiments).